Neuromedin U Depolarizes Rat Hypothalamic Paraventricular Nucleus Neurons In Vitro by Enhancing $I_{H}$ Channel Activity

De-Lai Qiu, Chun-Ping Chu, Tetsuro Shirasaka, Takashi Nabekura, Takato Kunitake, Kazuo Kato, Masamitsu Nakazato, Takahiko Katoh, and Hiroshi Kannan

Departments of 1Physiology, 2Anesthesiology, and 3Internal Medicine, 4Public Health, Miyazaki Medical College, 5200 Kihara, Kiyotake, Miyazaki 889-1692, Japan

Submitted 10 March 2003; accepted in final form 11 April 2003


INTRODUCTION

Neuromedin U (NMU) is a neuropeptide that is present in the gut and CNS (Ballesta et al. 1988; Minamino et al. 1985) and has potent effects on smooth muscle. The two receptors for NMU, NMU-R1 and NMU-R2, are G protein-coupled receptors (GPCRs) (Howard et al. 2000). NMU-R2 is expressed in the paraventricular nucleus (PVN) of the rat hypothalamus (Raddatz et al. 2000). NMU induces extracellular acidification, arachidonic acid metabolite release, and intracellular Ca$^{2+}$ (Raddatz et al. 2000). NMU induces extracellular acidification, the paraventricular nucleus (PVN) of the rat hypothalamus (Monteggia et al. 2000). Four different isoforms of the $I_{H}$ channel have been cloned, and two different isoforms (HCN1 and HCN3) are highly expressed in rat PVN (Monteggia et al. 2000).

Because $I_{H}$ channels and NMU receptors are both present in rat PVN, the goal of the present study was to determine the effect of NMU activation on $I_{H}$ channels and PVN neuron activity. We used a whole cell patch-clamp method to examine the effects of NMU on rat PVN neurons in vitro.

METHODS

Hypothalamic slice preparation

Hypothalamic slices were prepared from P12- to P14-day-old male Wistar rats, as previously described (Shirasaka et al. 2001). All experiments were approved by the Ethics Committee of the Miyazaki Medical College and were in accordance with international guidelines on the ethical use of animals in laboratory experiments. Briefly, the brain was quickly removed and placed into ice-cold artificial cerebrospinal fluid (ACSF) consisting of (in mM) 140 NaCl, 3 KCl, 1.3 MgSO$_4$, 1.4 NaH$_2$PO$_4$, 11 d-glucose, 5 HEPES, 2.4 CaCl$_2$, and 3.25 NaOH. The pH was 7.3; the osmolarity was 290–300 mOsm, and the fluid was bubbled with 100% O$_2$. Coronal slices were 250 μm in thickness, including PVN, and were prepared using a vibrating brain slicer (DSE-2000; Duskaya, Kyoto, Japan). The slices were incubated for 1 h in a chamber filled with equilibrated ACSF at room temperature (24–26°C) before recordings were started.

Electrophysiology

Patch pipettes were made with a puller (PB-7; Narishige, Tokyo) from thick-wall borosilicate glass (GD-1.5; Narishige). They were filled with a solution consisting of (in mM) 130 potassium gluconate, 10 potassium acetate, 10 HEPES, 2.5 MgCl$_2$, 0.5 CaCl$_2$, and 10 NaCl. The pH was 7.3; the osmolarity was 290–300 mOsm, and the fluid was bubbled with 95% O$_2$/5% CO$_2$. Coronal slices were 250 μm in thickness, including PVN, and were prepared using a vibrating brain slicer (DS-2000; Duskaya, Kyoto, Japan). The slices were incubated for 1 h in a chamber filled with equilibrated ACSF at room temperature (24–26°C) before recordings were started.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests: H. Kannan, Department of Physiology, Miyazaki Medical College, 5200 Kihara, Kiyotake-cho, Miyazaki-gun, Miyazaki 889-1692, Japan (E-mail: kannanh@post.miyazaki-med.ac.jp).
HEPES, 10 KCl, 1 CaCl₂, 5 EGTA, 1 MgCl₂, 2 Na₂ATP, and 0.5 Na₃GTP. The pH was adjusted to 7.2 with KOH. Patch pipette resistances were 5–7 MΩ in the bath, with series resistances in the range of 10–20 MΩ, compensated by 80%. The liquid junction potential (10 mV) was corrected for according to the method described by Neher (1992). Membrane potentials and/or currents were monitored with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), acquired through a Digidata 1200 series A/D interface on a personal computer using Clampex 7.0 software (Axon Instruments). Selected traces were saved to the hard drive of a computer, and all data were saved to a 4.7-GB DVD-RAM.

The membrane potential and current were low-pass-filtered at 1–5 KHz. Whole cell recordings were made from microscopically identified cells. Once stable recording conditions were obtained, a PVN neuron was identified electrophysiologically as type I (magnocellular) or type II (parvocellular) according to previously established criteria by current-clamp in standard ACSF (Luther et al. 2000). Type I neurons displayed transient outward rectification, while Type II did not. In voltage-clamp, TTX was routinely included in external recording solutions to block voltage-gated Na⁺ channels.

Chemicals

Reagents included rat NMU-23 (Peptide Institute, Japan), ZD 7288 (Tocris Cookson, Ballwin, MO), CsCl (Sigma, St. Louis, MO), and TTX (Sigma). ZD 7288 was prepared as a 50 mM stock solution (in H₂O) and stored at −20°C until use. The other drugs were dissolved in ACSF.

Data analysis

Data were analyzed using Clampfit 8.0 (Axon Instruments) and are expressed as mean ± SE. I₇ was determined by subtracting I₈ from I₉ at each hyperpolarizing voltage step using the equation

\[ I₇ = I₉ - I₈ \]

and I₇ conductance (G₇) was estimated as the amplitude of I₇ measured at various potentials (V) divided by the driving force (V − E₇), where E₇ is the reversal potential of I₇ (Ghamari-Langroudi and Bourque 2000) as follows

\[ G₇ = \frac{I₇}{V - E₇} \]

Differences between mean values recorded under control and test conditions were evaluated using Student’s t-test or one-way ANOVA with Tukey’s post-hoc test. Differences were considered significant at \( P < 0.05 \).

RESULTS

Neuronal membrane properties

A total of 309 PVN neurons (66 type I, 243 type II) were sampled under whole cell current-clamp conditions. While none of the type I neurons showed a response to NMU, 76 of the type II neurons (31%) were sensitive to NMU. These neurons expressed a depolarizing response approximately 1 min after application of 100 nM NMU; specifically, they displayed time-dependent inward rectification during the hyperpolarizing pulses (Fig. 1, C and D) that was blocked by 70 μM ZD 7288 (Fig. 1B) or 3 mM Cs⁺ (not shown). These characteristics are consistent with I₇ conductance (Ludwig et al. 1998; Santoro et al. 1998). Further, the responsive neurons exhibited a lack of transient outward rectification in response to a series of depolarizing current pulses delivered at a hyperpolarized membrane potential (Fig. 1A) (Luther et al. 2000).

FIG. 1. Electrophysiological properties of paraventricular nucleus (PVN) neuromedin U (NMU)-sensitive neurons. A: the neuron displayed time-dependent inward rectification and lacked transient outward rectification (black arrow) in response to a series of depolarizing current pulses delivered at a hyperpolarized membrane potential. B: the neuron displayed inward rectification which was blocked by 70 μM ZD 7288 (Fig. 1B). C: the neuron responded to a series of hyperpolarizing current pulses, expressing inward rectification. D: current-voltage relationships were obtained at the peak voltage (○) and at the steady-state voltage (■) during the hyperpolarizing pulses.
Effects of NMU on membrane potential

Applications of NMU in concentrations ranging from 10 nM to 1 μM NMU resulted in depolarization and increased firing rate in a concentration-dependent manner in NMU-sensitive PVN neurons when the holding potentials were −60 mV (Fig. 2); however, the characteristics of the action potentials were not changed (not shown). The depolarization response appeared approximately 40 s after NMU exposure and peaked at approximately 100 s, with a maximal depolarization range of 1.75 ± 0.47 to 7.18 ± 1.21 mV (Fig. 2C). The minimum NMU dose required to elicit an effect on membrane potential was 1 nM, and the maximum dose was approximately 1 μM. The EC50 was approximately 70 nM. This response was unaffected by the presence of 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) + 10 μM bicuculline (7.18 ± 1.21 mV in the presence of 1 μM NMU; mean ± SE, P > 0.05, n = 5).

Effects of NMU on membrane current

Application of 1 μM NMU to NMU-sensitive neurons with voltage-clamp at −60 mV produced a negligible inward current (7.5 ± 2.1 pA, n = 16). However, when neurons were held at −60 mV and a series of 1-s hyperpolarizing voltage steps were held from −60 to −150 mV, NMU induced a significant increment in steady-state current (Iss) at step potentials less than −80 mV (Fig. 3, A and B, P < 0.05, n = 7) and instantaneous current (Iinst) at step potentials less than −100 mV (Fig. 3, A and C, P < 0.05, n = 7). NMU-induced increases in Iinst was not affected by Ba2+ (Fig. 4A), but was blocked by ZD 7288 (Fig. 5C) or Cs+ (not shown). Simultaneously, the net reversal potential of the NMU-sensitive component of the fast current (determined in the presence of 100 μM Ba2+) was compared with the reversal potential for Ih (also determined in the presence of 100 μM Ba2+) (Cardenas et al. 1999). As illustrated in Fig. 4, A and B, the reversal potential was −34.0 ± 2.6 mV (n = 4) under control and NMU conditions. This was similar to the reversal potential of −33.5 ± 2.1 mV (n = 4) obtained with another group of NMU-sensitive neurons for the portion of Ih which was increased by changing the holding potential from −60 to −80 mV in the presence of Ba2+ (Fig. 4, C and D). Thus the increase in Ih produced by the shift in holding potential likely reflects an increase in tonically open Ih channels (Mayer and Westbrook 1983).

Blockade of the effects of NMU by ZD 7288 or Cs+

In current clamp, NMU-induced depolarization was not affected by TTX (Fig. 5A). However, application of ZD 7288 induced slight hyperpolarization in NMU-sensitive PVN neu-

![Figure 2](http://jn.physiology.org/)

**FIG. 2.** Effects of NMU on PVN NMU-sensitive neurons in current-clamp. A1, A2, and A3 are the responses to 10 nM, 100 nM, and 1 μM NMU, respectively (bar, Vh = −60 mV). B1, B2, and B3 are the instantaneous spike rates of the neurons in A1, A2, and A3, respectively. NMU elicited increases in firing-action potential in a dose-dependent manner. C: the mean time courses of NMU-induced depolarizations. Note that NMU induced dose-dependent depolarization. (+) P < 0.05 vs. ACSF, (⁎) P < 0.05 vs. 10 nM NMU, (#) P < 0.05 vs. 100 nM NMU. D: the concentration-response curve of NMU-induced depolarization. The EC50 value obtained from the curve was approximately 70 nM. The number of neurons tested for each concentration is indicated near the bars.
rons and abolished NMU-induced depolarization (Fig. 5B). NMU increased I_{ss} and I_{ins} according to hyperpolarizing pulses. To determine whether NMU-induced increase in I_{ss} and I_{ins} represented enhanced I_{H} channels, ZD 7288 and Cs" (not shown) were used as I_{H} channel blockers (Harris and Constanti 1995; Maccaferri and McBain 1996; McCormick and Pape 1990; Pape 1996). ZD 7288 significantly blocked I_{ins} and NMU-induced increments of I_{ins} at step potentials less than −80 mV. The I-V relationships were linear in the presence of ZD 7288 (Fig. 5, C and D). Further, ZD 7288 blocked I_{ss} and NMU-induced increments of I_{ss} at step potentials less than −80 mV. The I-V relationships were also linear in the presence of ZD 7288 (Fig. 5, C and E). This finding indicates that NMU-sensitive neurons have I_{H} channels, which produce hyperpolarization-activated ZD 7288-sensitive inward currents (I_{H}) and are activated at RMP, thus producing ZD 7288 sensitive I_{ins}.

Effects of NMU on I_{H}

In this study, 1 μM NMU significantly enhanced I_{H} activity at step potentials less than −80 mV, and the maximal effects were at step potentials of −100 to −120 mV (Fig. 3D). Furthermore, we estimated the effect of NMU on I_{H} conductance (G_{H}) (see METHODS). E_{H} was obtained in the NMU-sensitive neurons as shown in Fig. 6A: following a step to −120 mV (1-s duration), the membrane voltage was stepped in the range of −110 to −50 mV (1-s duration, 10-mV increments) (Maccaferri and McBain 1996). The plot of the instantaneous current at each test potential yielded the fully activated I-V relationship, which was linear (Fig. 6B). The extrapolated reversal potential (E_{H}) was −33.1 ± 1.8 mV (Fig. 6B, n = 5), similar to that previously reported for E_{H} in other nervous preparations (Maccaferri and McBain 1996; McCormick and Pape 1990; Pape 1996). The mean G_{H}V_{r} relationships are shown in Fig. 6C. Note that NMU enhanced I_{H} conductance at step potentials more negative than −80 mV (P < 0.05, n = 7). Furthermore, the modified Boltzmann equation was used as follows

\[
G_{H(V)} = 1/(1 + e^{(V-V_{H}/k)})
\]

where G_{H(V)} is the fraction of maximal G_{H} observed at V, k is the slope factor, and V_{1/2} is the half-maximal voltage. The mean values were as follows: V_{1/2} = −110.2 ± 2.3 mV, k = 13.1 ± 2.0 in control, and V_{1/2} = −99.5 ± 3.4 mV, k = 11.5 ± 1.9 during the application of NMU. These data reveal that NMU produced a significant shift in V_{1/2} to a more depolarized potential (Fig. 6C, insert; P < 0.05). The slope factor values were not altered by NMU (P > 0.05).

Effects of NMU on the kinetics of I_{H} activation

The time course of activation of I_{H} was obtained from an analysis of the rising phase of the NMU-induced I_{H} current evoked by hyperpolarizing steps to various voltages. As shown in Fig. 6D, the I_{H} current traces were fit to a single exponential function of the form \( A_{i} = A_{s}(1 - e^{-t/\tau}) \), where \( A_{i} \) is the amplitude of I_{H} at time t, \( A_{s} \) is the amplitude of I_{H} at a steady state, and \( \tau \) is the activation time constant (Ghamari-Langroudi
NMU enhancing $I_{\text{H}}$ and exciting PVN neurons

DISCUSSION

This study demonstrated that NMU enhanced $I_{\text{H}}$ channels activity leading to excitatory responses in a subpopulation of PVN type II neurons.

Expression of $I_{\text{H}}$ in PVN NMU-sensitive neurons

In the present study, NMU-sensitive PVN neurons displayed a time-dependent strong inward rectification during hyperpolarizing pulses (Fig. 1, C and D) that was blocked by 70 μM ZD 7288 (Fig. 1B). Further, these neurons did not display transient outward rectification. These properties are consistent with $I_{\text{H}}$ conductance produced by $I_{\text{H}}$ channels (Ludwig et al. 1998; Luther et al. 2000; Santoro et al. 1998; Stern 2001; Tasker and Dudek 1991).

NMU excited PVN NMU-sensitive neurons by enhanced $I_{\text{H}}$

NMU evoked small depolarization and increased neuronal excitability. Several previous studies have demonstrated that enhancement of $I_{\text{H}}$ results in increased neuronal excitability and responsiveness to excitatory input. This occurs via release of neurons from tonic hyperpolarizing synaptic input and via facilitation of action potential triggering by depolarizing input or counter-balancing after-hyperpolarizations following action potentials (Pape 1996; Pape and McCormick 1989; Yagi and Sumino 1998). The presence of $I_{\text{H}}$ in magnocellular neurosecretory cells of rat supraoptic nucleus provides an excitatory drive that contributes to phasic and tonic firing (Ghamari-Langroudi and Bourque 2000). $I_{\text{H}}$ plays a significant role in setting both the RMP and the baseline level of excitability of hippocampal GABAergic interneurons found in the stratum oriens of area CA1 (Lupica et al. 2001).

Several findings in the current study suggest that NMU excites PVN neurons via enhanced $I_{\text{H}}$ channel activity. First, the RMP of NMU-sensitive neurons was approximately −58 mV ($V_h = −60$ mV), and the $E_{\text{H}}$ was approximately −33 mV (Fig. 6B), suggesting that $I_{\text{H}}$ channels are partially active at RMP (Yagi and Sumino 1998). NMU enhanced the activity of $I_{\text{H}}$ channels at RMP, induced NMU-sensitive neurons depolarizing in current-clamp (Fig. 2), and evoked increments of $I_{\text{Ins}}$ by hyperpolarizing steps (Fig. 3C). NMU-induced increment in $I_{\text{Ins}}$ was almost completely blocked by ZD 7288 (Fig. 5C), but not by Ba$^{2+}$ (Fig. 4, A and B). Thus this likely reflects an increase in tonically activated $I_{\text{H}}$ (Mayer and Westbrook 1983). Second, in voltage clamp, NMU resulted in an increase of $I_{\text{H}}$ current and enhanced channel kinetics at step potentials less than −80 mV. NMU also produced a significant shift in $V_{1/2}$ to a more depolarized potential. Third, ZD 7288 completely blocked NMU-induced depolarization (Fig. 5B) and abolished the effects of NMU on the neurons in voltage clamp (Fig. 5, C,

- Figure 6D2 reveals the plots of the means ± SE (n = 7) against voltage steps. The $\tau$ of NMU-sensitive neurons decreased from 800 ms at −70 mV to 100 ms at −140 mV and exhibited fast kinetics. NMU enhanced $I_{\text{H}}$ channels kinetics exhibiting decrements of $\tau$ at step potentials more negative than approximately −80 mV ($P < 0.05$ vs. ACSF, n = 7).

- Figure 4. Determination of reversal potentials for the fast current increased by NMU or hyperpolarization in the presence of 100 μM Ba$^{2+}$. A: current traces elicited by a series of 100-ns hyperpolarizing voltage steps (10-mV decrement, holding potential was −60 mV) in the presence of 100 μM Ba$^{2+}$ (A1) and in the presence of 100 μM Ba$^{2+}$ + 1 μM NMU (A2). B: plots of fast current amplitude vs. command voltage for the currents shown in A1 and A2: 100 μM Ba$^{2+}$; 100 μM Ba$^{2+}$ + 1 μM NMU. Straight lines were fitted to the data points using best fit values for slope and intercept determined by linear regression. The theoretical reversal potential for NMU increased fast current, indicated by the intersection of 2 lines, was extrapolated to −36 mV. C: families of currents evoked in the same cell as depicted in A, using 750-ns hyperpolarizing commands ranging from −70 to −120 mV from a $V_h$ of −60 mV (C1) and −80 mV (C2), in the presence of 100 μM Ba$^{2+}$. D: plots of fast current amplitude vs. command voltage for the current shown in C1 and C2: 100 μM Ba$^{2+}$, $V_h = −60$ mV; 100 μM Ba$^{2+}$, $V_h = −80$ mV. Straight lines were fitted to the data points for $V_h$ values as described for B. The theoretical reversal potential for the hyperpolarization increased fast current was −34 mV, as estimated from straight lines fitted to the data.
However, NMU did not induce large depolarization, likely because \( I_H \) maintains the membrane potential of neurons within the range necessary for the generation of tonic action potential firing (Ghamari-Langroudi and Bourque 2000; Williams et al. 2002). Recently, it was reported that icv administration of NMU induced c-fos expression in magnocellular cells (type I) and parvocellular cells (type II) (Ozaki et al. 2002). The expression of the c-fos gene in the PVN should reflect the neural activation either directly or indirectly after icv administration of NMU (Ozaki et al. 2002). NMU-R2 is expressed in the PVN of the hypothalamus, along the wall of the third ventricle in the hypothalamus (Howard et al. 2000). In this study, NMU-induced responses in membrane potential were unaffected by TTX + CNQX + bicuculline. This evidence suggests that NMU depolarizes PVN type II neurons via a direct postsynaptic action rather than by indirect modulation of neurotransmission. On the other hand, none of the type I neurons showed a response to NMU. According to our data, we suggest that the expression of the c-fos gene in the PVN magnocellular may be mainly via an unknown indirect pathway after icv administration of NMU.

**Possible mechanism of NMU action**

NMU-R2 is expressed in the PVN of the rat hypothalamus (Howard et al. 2000), and HCN1 and HCN3 mRNA expression is highly enriched in the PVN (Monteggia et al. 2000). It is possible that PVN NMU-sensitive PVN neurons contain \( I_H \) (HCNs) channels and NMU-R2. A key property of neuronal HCN channels is their regulation by neurotransmitters and hormones that act via cAMP, cGMP, or intracellular Ca\(^{2+}\) (Pape 1996). The cAMP and cGMP affect HCN channels by increasing cAMP (Lu¨thi and McCormick 1998). NMU-R1 is coupled to phospholipase C stimulation via a \( G_q \)-type G protein, resulting in the release of the inositol phosphate (IP) second messenger and increased intracellular Ca\(^{2+}\) in COS-7 cells (Raddatz et al. 2000). NMU-R2 is also coupled to the \( G_q \) family of G proteins in cells, inducing a rapid increase in intracellular Ca\(^{2+}\) (Shan et al. 2000). In thalamic neurons, transient increases in intracellular Ca\(^{2+}\) appeared to cause a reversible augmentation of \( I_H \) attributable to the rapid, Ca\(^{2+}\)-dependent formation of cyclic nucleotides (Lu¨thi and McCormick 1998). The increment of intracellular Ca\(^{2+}\) activates soluble guanylate cyclase, leading to increased cGMP levels (Kuzmiski and Macvicar 2001).

Thus we propose that NMU binds to NMU-R2, resulting in
increased intracellular calcium and cGMP. This leads to an increase in $I_{\text{H}}$ current and neuronal excitation. This response may contribute to activation of autonomic centers in the brain stem and spinal cord that regulate MABP, HR, and plasma norepinephrine.

**DISCUSSIONS**

This work was supported in part by a Grant-in-Aid for Science Research (14370024) from the Ministry of Education, Science, Sports, and Culture, Japan, and the Japanese Center of Excellence Program (Section of Life Sciences). This study was also carried out as part of the “Ground Research Announcement for Space Utilization” promoted by the Japan Space Forum.

**REFERENCES**


Williams SR, Christensen SR, Stuart GJ, and Hausser M. Membrane potential bistability is controlled by the hyperpolarization-activated current I_{Na} in rat cerebellar Purkinje neurons in vitro. J. Physiol. 539: 469–483, 2002.